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Differential Effects of Parainfluenza Virus Type 3 on Human Monocytes and Dendritic Cells

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To understand the lack of protective immunity observed after infection with parainfluenza virus type 3 (PIV3), we tested the effect of the virus on human monocytes and monocyte-derived immature dendritic cells (DCs). Expression of viral antigens on the cell surfaces correlated with replication of the virus, which was marginal in monocytes but extremely efficient in DCs. The virus increased monocyte survival at least in part through the production of granulocyte–macrophage colony-stimulating factor but, in contrast, accelerated DC apoptosis. In addition, PIV3 infection failed to activate monocytes but induced maturation of DCs with increased expression of CD54, HLA-DR, CD86, and CD83 and production of bioactive IL-12. However, PIV3-infected DCs demonstrated low stimulatory properties in DC–T cell cocultures, a finding that could not be attributed to the production of infectious virus or IL-10. These results demonstrate for the first time that PIV3 dramatically modifies the survival and/or the function of antigen-presenting cells and might therefore prevent the development of efficient antiviral immune responses.

INTRODUCTION

Parainfluenza virus type 3 (PIV3) is a major respiratory tract pathogen responsible for bronchiolitis, pneumonia, and croup (Reed *et al.,* 1997). Initial infection occurs during infancy and early childhood, with the peak incidence of severe illness between 4 and 12 months of age. Reinfection is a common event and may occur several times, even in adolescents and adults (Marx *et al.,* 1999; Welliver *et al.,* 1982), although clinical manifestations become progressively more restricted to the upper respiratory tract. The repeated occurrence of PIV3 infection cannot be explained by antigenic shift of the virus, which is relatively stable (Chanock *et al.,* 1961). Furthermore, persistence of PIV3 has been documented in various situations (Goswami *et al.,* 1984; Muchmore *et al.,* 1981). Consequently, and in marked contrast with epidemic patterns of other respiratory infections, PIV3 may be recovered almost continuously in the human population (Chanock *et al.,* 1961; Glezen *et al.,* 1971).

The ability to reinfect, within a short time interval, the same individual and to cause persistent infection suggests that PIV3 fails to induce a state of long-lasting immunity. Although the respiratory disease and the length of virus shedding seem to be reduced in the presence of high titers of neutralizing antibodies (Chanock *et al.,* 1961), activation of T cell responses may be essential in recovery, since the most severe infections occur in individuals with defects in cell-mediated immunity (Dorman *et al.,* 1999; Fishaut *et al.,* 1980) or with severe combined immunodeficiency disease (Taylor *et al.,* 1998).

Previous studies have demonstrated that PIV3 profoundly impairs T cell functions *in vitro.* The virus infects activated T lymphocytes and significantly decreases their proliferative capacity (Sieg *et al.,* 1994). IL-10 produced by blood mononuclear cells maintains these infected cells in a nonresponsive state. In addition, T cell cytotoxicity is prevented by selective inhibition of granzyme B mRNAs (Sieg *et al.,* 1995).

The development of antiviral T cell responses relies on specific interactions between naive T cells and professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and monocytes/macrophages. Although less potent than DCs in stimulating naive T cells, monocytes are involved in the restimulation of memory and effector T cells, which have less of a requirement for cosignals and accessory molecules (Diaz *et al.,* 1997). In contrast, DCs are crucial for the initiation of a primary response. Immature DCs are located in most nonlymphoid tissues, including the respiratory mucosa, where they capture, process, and carry foreign antigens (Ags) to the secondary lymphoid organs (Banchereau *et al.,* 1998; Cella *et al.,* 1997; Steinman *et al.,* 1999). During their migration, DCs are thought to undergo modulation of both phenotype and function, a process referred to as DC maturation. They express high levels of costimulatory molecules (such as CD40, CD54, CD58, CD86, and MHC class I/II), become CD83-positive, and lose their ability to capture Ags. The resulting mature DCs activate naive T cells and

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stimulate primary T cell responses. In addition, they produce cytokines such as IL-12 that are critically involved in the generation of Th1 responses and the generation of cellular immunity (Trinchieri, 1998). However, a variety of pathogens have been shown to be able to infect professional APCs and either to induce or to inhibit their stimulatory properties (Cella *et al.,* 1999; Engelmayer *et al.,* 1999; Klagge and Schneider-Schaulies, 1999; Salio *et al.,* 1999).

Since induction of T cell immunity via activation of professional APCs may be central in the control of PIV3 infection, the aim of this study was to examine the effect of PIV3 on human monocytes and DCs. Our results demonstrate that PIV3 differentially affects these cells. While it increases the survival of monocytes and replicates poorly in these cells, it's replication is massive and highly toxic for DCs. Furthermore, although maturation of DCs is induced with the production of IL-12, the infected cells demonstrate low stimulatory properties.

RESULTS

Replication of PIV3 in DCs and monocytes

Expression of viral antigens on the surfaces of monocytes and DCs was determined by FACS analysis. These antigens were expressed at very low levels on the whole monocyte population after infection at a m.o.i. of 10 (Figs. 1A and 1B). In contrast, they were detected from day 1 (16 h) on DCs and increased in a time and dose-dependent manner to reach a maximum at day 2 of a m.o.i. of 10, at which stage most of the cells were positive (Figs. 1C and 1D). As expected, PIV3 antigens were undetectable on mock-infected monocytes and DCs or on these cells previously incubated with UV-inactivated PIV3 (not shown).

The infectious PIV3 yield was determined in the supernatants of these cells. As indicated in Table 1, PIV3 was always detected at levels close to the limit of detection of the assay in cultures of infected monocytes (from 0.70 to 1.33 $\log_{10}$ $TCID_{50}$/ml), even when infection was performed after previous stimulation of these monocytes with LPS or CD40 ligand (not shown), attesting to the poor replication rate of PIV3 in these cells. In contrast, PIV3 was present at high levels from 16 h onward in DC cultures and with more than 5.20 $\log_{10}$ $TCID_{50}$/ml by day 1, at all m.o.i.s tested. No virus was detected in control cultures or in cultures after infection with UV-inactivated virus ($<$0.45 $TCID_{50}$/ml).

Thus, in agreement with cell surface expression of viral antigens, PIV3 replicates marginally in monocytes but very efficiently in DCs.

PIV3 increases monocyte survival and accelerates DC apoptosis

The effect of PIV3 infection on monocytes and DCs was investigated by determination of cell survival.

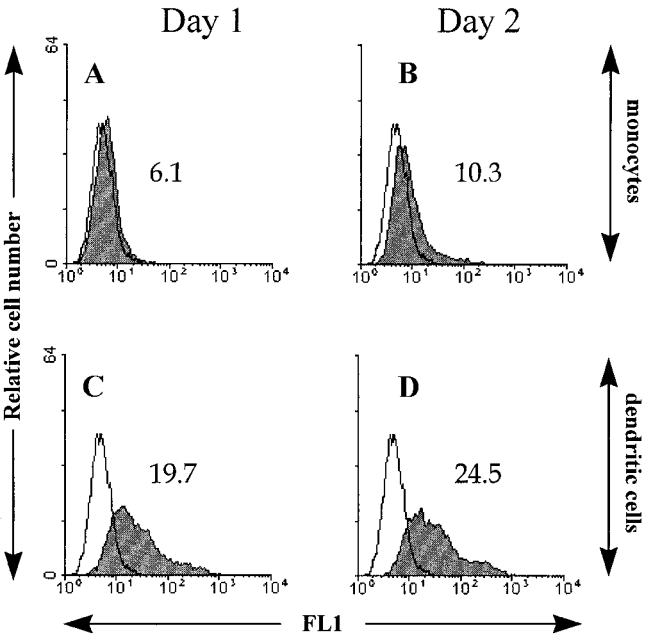

FIG. 1. Expression of PIV3 antigens on monocytes and DCs. Monocytes and DCs were mock-infected or exposed to PIV3. Expression of viral antigens was determined by FACS analysis 1 and 2 days after infection of monocytes (A and B, respectively) or DCs (C and D, respectively) with PIV3 of a m.o.i. of 10. Data (MFI) show infected versus uninfected cells after staining with anti-PIV3 antiserum. None of these cells was stained with the control antiserum (not shown). Results are representative of one of five separate experiments.

Monocytes progressively died in all cultures from day 1 onward by an apoptotic-related mechanism (Mangan and Wahl, 1991). At day 3, the proportion of live cells in mock-infected cultures was lower than 40% (Fig. 2A). As expected (Mangan and Wahl, 1991), cell survival increased in the presence of exogenous granulocyte–macrophage colony-stimulating factor (GM-CSF) (Fig. 2B). Surprisingly, infection with PIV3 decreased monocyte apoptosis in a dose-dependent manner with an effect significant at a m.o.i. of 1 and maximal at a m.o.i. of 5 or 10: more than 75% of monocytes were still alive 72 h after infection with PIV3 at a m.o.i. of 10 (Fig. 2C). Furthermore, this effect was also induced after incubation of monocytes with UV-inactivated virus (not shown), indicating that virus–cell interactions were involved but that the increased monocyte survival was independent of viral replication. In contrast to monocytes, infection of immature DCs with PIV3 resulted in a significant increase in cell apoptosis (Figs. 2E and 2F), except when PIV3 had been previously UV-inactivated (not shown). More than 60% of DCs were dead or undergoing apoptosis at day 3 after infection with PIV3 at a m.o.i. of 5 (Fig. 2E) and almost no cells survived at a m.o.i. of 10 (Fig. 2F).

Thus, infectious PIV3 induced opposite effects on monocytes and DC programmed cell death, favoring

TABLE 1

PIV3 Replication in Cultures of DCs and Monocytes

	m.o.i.				
	None	1	5	10	10 (inactivated)
Monocytes					
Day 1	<0.45	≤0.70 ± 0	≤0.76 ± 0.37	≤1.33 ± 1.20	<0.45
Day 2	<0.45	<0.45	≤0.9 ± 0.37	<0.45	<0.45
Day 3	<0.45	<0.45	≤0.86 ± 0.52	≤0.70 ± 0	<0.45
DCs					
16 h	<0.45	≤2.95 ± 0.35	≤3.95 ± 0.70	nd	<0.45
Day 1	<0.45	>5.20	>5.20	>5.20	<0.45
Day 2	<0.45	>5.20	>5.20	>5.20	<0.45
Day 3	<0.45	>5.20	>5.20	>5.20	<0.45

Note. Monocytes and immature DCs were incubated or not with live or UV-inactivated PIV3 at a m.o.i. of 1, 5, or 10. Supernatants were collected after 1, 2, or 3 days of culture and PIV3 titers calculated. Results are expressed as $\log_{10}$ $TCID_{50}$ of PIV3/ml and are representative of four separate experiments. nd, not done.

monocyte survival and accelerating immature DC apoptosis.

PIV3 increases monocyte survival by induction of GM-CSF

Apoptosis of blood monocytes can be prevented by a number of cytokines, including GM-CSF (Mangan and Wahl, 1991). Indeed, GM-CSF mRNA was significantly induced in PIV3-infected monocytes at a m.o.i. of 5 (Fig. 3A, lane 3) as well as in monocytes previously incubated with UV-inactivated virus (Fig. 3, lane 4). To determine whether GM-CSF was also translated in these cells and could play a role in the increased survival of infected monocytes, an anti-GM-CSF neutralizing Ab was added into the PIV3-infected cultures at day 0. As represented in Fig. 3B, infection of monocytes at a m.o.i. of 5 signifi-

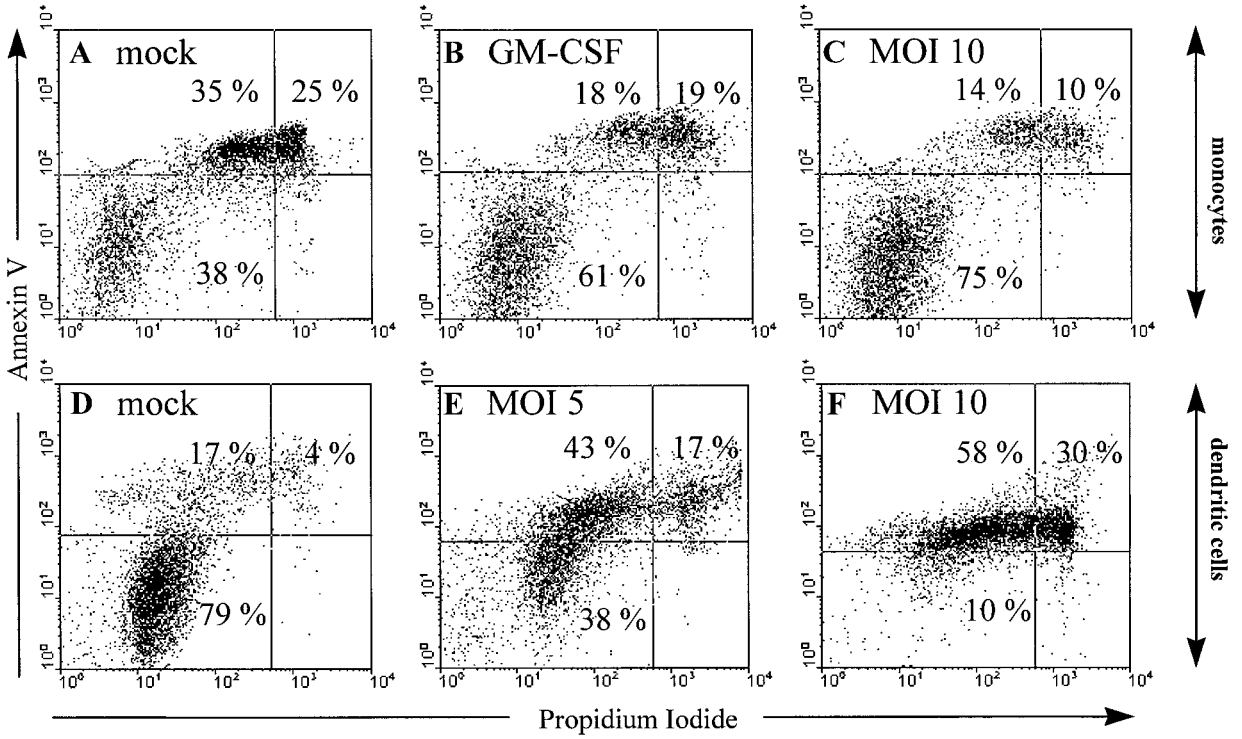

FIG. 2. Dual effects of PIV3 on apoptosis of monocytes and DCs. Monocytes and immature DCs were mock-infected or exposed to PIV3. Cell viability and apoptosis were evaluated at 72 h in cultures containing uninfected monocytes alone (A), monocytes cultured in the presence of GM-CSF (B), monocytes infected with PIV3 at a m.o.i. of 10 (C), uninfected DCs (D), and DCs infected at a m.o.i. of 5 (E) and at a m.o.i. of 10 (F). Data are representative of one of six separate experiments.

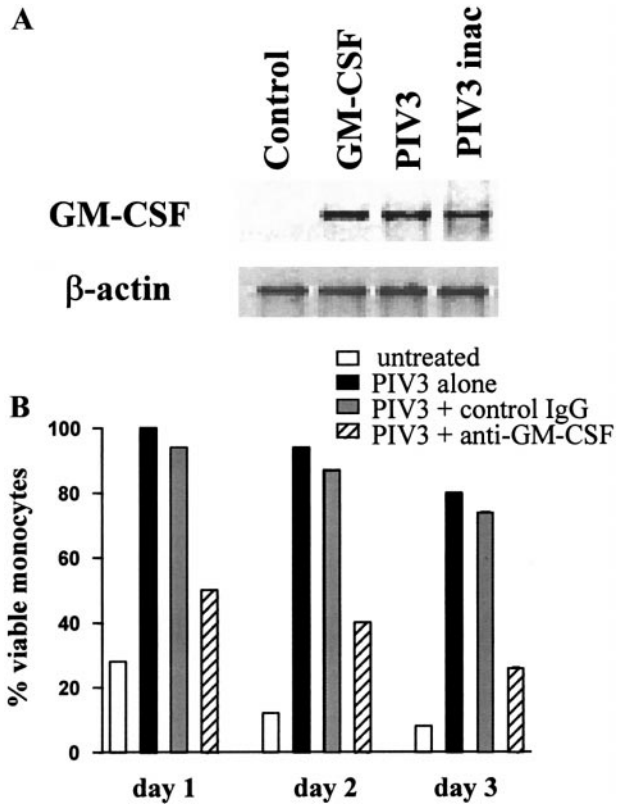

FIG. 3. The role of GM-CSF in the increased survival of PIV3-infected monocytes. Monocytes were mock-infected, exposed to infectious or UV-inactivated PIV3, and cultured for 2 days. (A) PCR amplification of β-actin and GM-CSF mRNAs in monocytes cultured alone (lane 1), in the presence of GM-CSF (lane 2), or after incubation with infectious (lane 3) or inactivated (inac) PIV3 at a m.o.i. of 5 (lane 4). (B) The % of viable cells 1, 2, or 3 days after culture of monocytes previously infected at a m.o.i. of 5 and cultured in the presence of 20 μg/ml of anti-GM-CSF or control Ab in one representative experiment of three.

cantly increased the number of viable cells compared to that in mock-infected monocytes. The anti-GM-CSF Ab reduced the number of viable monocytes by an average of 50% from day 1 to day 3, with similar effects at 10 or 20 μg/ml. An isotype-matched control Ab had no significant effect. Thus, PIV3 increases the survival of monocytes at least in part through the induction of GM-CSF.

PIV3 induces DC maturation but does not affect monocyte activation

Since virus infection may induce maturation of APCs (Cella *et al.,* 1999; Iwasaki and Kelsall, 1999), we investigated the ability of PIV3 to activate monocytes and DCs. As represented in Table 2, increased expression of CD54, HLA-DR, and CD86 as well as induction of CD83 was evident on DCs after previous infection with PIV3, as was observed when DCs were cultured with TNF-α. In addition, these cells appeared veiled under light microscopy (not shown). These criteria correspond to DC maturation. Interestingly, these phenomenons were also observed with UV-inactivated PIV3, indicating that viral replication was not required but that cell interactions with virus particles were sufficient. Furthermore, a significant level of IL-12 was detected both in cultures of PIV3-infected DCs and in DCs after incubation with the UV-inactivated virus. In contrast, infectious and UV-inactivated PIV3 consistently failed to induce activation of monocytes, as indicated by low expression of CD54, CD80, CD86, CD58, and HLA-DR on these cells at all doses and days tested (not shown). Thus, although incubation with PIV3 does not induce monocyte activation, it is very effective at inducing a maturation-like phenotype in DCs.

PIV3-infected DCs display impaired stimulatory properties

The stimulatory properties of PIV3-infected DCs were evaluated in DC–T cell cocultures. Immature DCs were incubated with infectious or inactivated PIV3 and cultured for 2 days. Negative and positive control DCs for

TABLE 2

PIV3 Induces Maturation of DCs

			CD86[c]		CD83[c]		
Stimulus[a]	CD54[b]	DR[b]	%	MFI	%	MFI	IL-12[d] (ng/ml)
None	82	25	15	36	15	8	0.5
PIV3, m.o.i. 5	133	102	100	361	100	46	4.5
PIV3, m.o.i. 5 (inactivated)	180	118	100	270	43	27	2.2
TNF-α (20 ng/ml)	180	118	100	213	100	75	—
LPS (100 ng/ml)	—	—	—	—	—		5.6

Note. Data are representative of one of three separate experiments.

[a] Immature DCs were exposed to infectious or UV-inactivated PIV3 (m.o.i. of 5), to TNF-α, or to LPS. Three days later, the phenotypes of the DCs were determined by FACS analysis as indicated under Materials and Methods.

[b] Results are expressed in MFI values after substraction of the values obtained with control mAbs.

[c] Results show the percentages and the MFI values of the positive cells.

[d] Results show the level of IL-12 in ng/ml obtained in culture of DCs 2 days after stimulation.

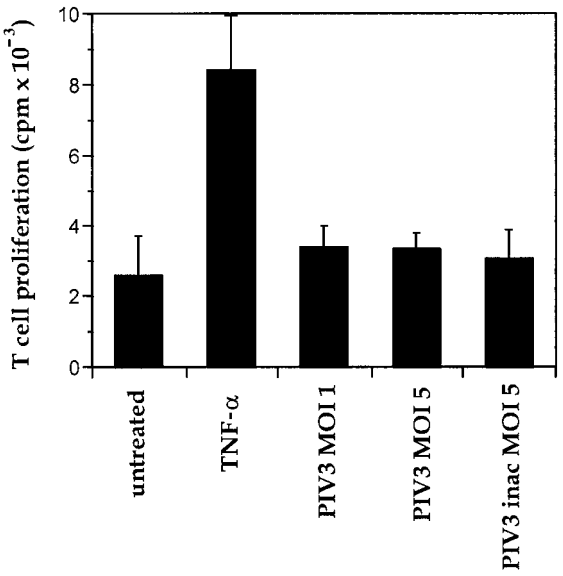

FIG. 4. PIV3-infected DCs display poor stimulatory properties. Immature DCs were exposed to infectious or UV-inactivated (PIV3 inac) PIV3 at a m.o.i. of 1 or 5 and cultured for 2 days. Negative and positive control cells for maturation were obtained with immature DCs cultured in medium alone and immature DCs cultured in the presence of TNF-α, respectively. All DCs were then UV-irradiated and cultured for 3 days with allogenic T cells at a DC:T cell ratio of 1:4 as described under Materials and Methods. Results represent the mean cpm ± SD calculated with quadruplicate values from one representative experiment of five.

maturation were obtained with immature DCs cultured in medium alone and DCs cultured in the presence of TNF-α, respectively. In contrast to DCs cultured with TNF-α, PIV3-infected DCs failed to stimulate allogeneic T cell proliferation. As shown in Fig. 4, thymidine uptake at day 3 was similar in cultures containing mock-infected or PIV3-infected DCs (at a m.o.i. of 5 or 10). A similar effect was obtained at day 5 (not shown) and with DCs previously incubated with the UV-inactivated virus (Fig. 4). T cell proliferation was similarly inhibited after fixation of DCs with paraformaldehyde (not shown). Thus, despite up-regulation of costimulatory molecules, PIV3-infected DCs are poor stimulators of allogeneic T cell proliferation.

DISCUSSION

PIV3 causes repeated pulmonary infections thought to be due to the induction of ineffective immune responses. In this study, we demonstrate that PIV3 profoundly modifies the survival and/or the function of APCs and may thus hinder the establishment of a state of long-lasting immunity.

Immature DCs are highly susceptible to PIV3 infection, which leads to massive replication of the virus and induces cell apoptosis after a few hours. This indicates that DCs, which are present in the airway epithelium and which can be rapidly recruited at this site after exposure to various pathogens (Iwasaki and Kelsall, 1999; McWilliam *et al.,* 1996), are a major target of PIV3. Furthermore, these cells probably contribute to active dissemination of the virus. DCs have been shown to be susceptible to infection by other viruses such as HIV and measles. These viruses replicate at a relatively low level in these cells, except upon activation, membrane interactions with molecules such as CD40, and interaction with T lymphocytes (Granelli-Piperno *et al.,* 1999; Fugier-Vivier *et al.,* 1997). Therefore, the majority of viral replication *in vivo* probably occurs during cellular interactions. Indeed, DCs are thought to serve as a reservoir for these viruses and a vehicle for transmitting infection to lymphoid cells in the draining lymph nodes. In contrast, PIV3 replication in DCs is spontaneously efficient. It starts after a few hours and results in the rapid elimination of these cells. Unlike DCs infected with HIV or measles, it is likely that most PIV3-infected DCs die within the pulmonary tissues where they get infected and that only small numbers of DCs are able to reach the draining lymph nodes. Interestingly, cell susceptibility to PIV3 infection seems to correlate with the differentiation rather than the activation state of the cells, since we found that the virus replicates poorly in resting and activated monocytes, but replicates very efficiently after a 4-day culture of these monocytes with GM-CSF (i.e., in macrophages) (personal observation) or with IL-4 and GM-CSF (i.e., in immature DCs). These results suggest that different cell receptor(s) for the virus are expressed on monocytes, macrophages, and DCs and/or that the consequences of viral interactions with these receptor(s) differ with the state of cell differentiation.

Previously, Cella *et al.* (1999) demonstrated that DC activation by viruses such as influenza results in the induction of type I IFN, which triggers cellular resistance to cytopathic effects via upregulation of MxA. This increased resistance allows infected DCs to present the corresponding antigens and to induce protective immune responses. Accordingly, Bender *et al.* (1998) showed that influenza virus infection of DCs is nontoxic, as viral expression on these cells is sustained for more than 2 days with retention of viability. In our experiments, a strong induction of IFN-α in PIV3-infected DC could also be demonstrated by RT–PCR (personal observation). In addition, PIV3 replication was shown to be significantly reduced by IFN-α and MxA (Zhao *et al.,* 1996). However, the rapid destruction of DCs and intense production of infectious virus after infection indicates that transcription of IFN-α fails to induce protective levels of MxA and/or that the virus neutralizes the activity of either IFN-α or MxA.

As shown *in vitro,* although most DCs are rapidly eliminated by apoptosis, the surviving DCs may undergo rapid maturation and, in contrast to HIV- or measles-infected DCs (Chehimi *et al.,* 1994; Fugier-Vivier *et al.,* 1997), produce spontaneously increased levels of IL-12.

However, despite up-regulation of costimulatory molecules, PIV3-infected DCs display only poor stimulatory properties that fail to induce efficient T cell proliferation.

Previous work has demonstrated that incubation of peripheral blood mononuclear cells (PBMC) with PIV3 leads to the production of IL-10 (Sieg *et al.,* 1996). IL-10 is known to play an important immunoregulatory role in infection with viruses such as EBV, HIV, and CMV (De Waal Malefyt *et al.,* 1991; Redpath *et al.,* 1999) by decreasing antigen presentation and IL-2 secretion, reducing T cell blastogenesis and preventing T cell proliferation (De Waal Malefyt *et al.,* 1991; Taga and Tosato, 1992). In our study, the poor stimulatory effect of infected DCs in T cell cocultures was not attributable to the production of IL-10, which was indeed undetectable in infected DCs and monocyte cultures (data not shown). In addition, this effect was not due to other soluble factors nor to newly synthesized virions able to infect the T cells because all DCs were washed and UV-irradiated before inclusion in T cell cultures, and similar effects were observed with paraformaldehyde-fixed cells.

Such a discrepancy between the maturation-like phenotype and the poor stimulatory ability of these DCs has been previously described for tumor-infiltrating DCs and for DCs previously infected with measles virus (Kiertscher *et al.,* 2000; Grosjean *et al.,* 1997). For measles virus-infected cells, this phenomenon was attributed to the presence of undefined surface molecules because it was also observed with fixed cells.

A direct role for viral-encoded proteins is conceivable since purified influenza glycoproteins can directly modulate the activity of immune cells (Arora *et al.,* 1984; Arora and Houde, 1992). In addition, we demonstrated that UV-inactivated PIV3 has the capacity, like the infectious virus, to completely prevent T cell proliferation in mixed lymphocyte cultures and in cultures with mitogen (personal observation). This indicates that the blockade of the T cell response does not require T cell infection but that it can result directly from interactions of inert viral particles on the cell surface.

Monocytes/macrophages are known to be involved in immune regulation and are capable of both activating and suppressing lymphocyte function (Wing and Remington, 1977; Unahue and Allen, 1987; Chouaib and Fradelizi, 1982). Previous studies have demonstrated that bovine PIV3 significantly impairs the ability of alveolar macrophages to stimulate other immune effector cells (Adair *et al.,* 1992; Basaraba *et al.,* 1993). These results are in accordance with ours showing that expression of costimulatory molecules is not up-regulated on monocytes infected with the human virus. Thus, these cells do not express surface molecules required for efficient effector/memory T lymphocyte restimulation.

Interestingly, in contrast to other paramyxoviruses that are lytic to monocytes (Tropea *et al.,* 1995; Esolen *et al.,* 1995), PIV3 significantly increases the survival of these cells by the induction of an autocrine loop involving GM-CSF. This phenomenon is also obtained with the inactivated virus, confirming the observation that viral replication is not necessary to induce the remarkable effects of PIV3 on monocyte survival, DC maturation, and T cell proliferation, but that virus/cell surface interactions are sufficient.

The increased survival of monocytes following contact with the infectious virus could favor persistence of infected cells *in vivo.* In addition, as shown in cultures, it is conceivable that PIV3 replication also occurs sporadically *in vivo* and may be stimulated under the influence of unknown biological factors. Under these circumstances, monocytes could be responsible for PIV3 latency after primary infection and account for the resurgent infections that have been described in some individuals (Chanock *et al.,* 1961; Muchmore *et al.,* 1981).

Thus, we have demonstrated that PIV3 might prevent the development of efficient and long-lasting immune responses by modifying the critical steps required for the generation and the reactivation of T cell immune responses, while blood monocytes might constitute a potential *in vivo* reservoir for the virus.

MATERIALS AND METHODS

1. Monocyte isolation and DC generation

PBMC were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll–Hypaque (Pharmacia, Uppsalla, Sweden). Monocytes were purified from PBMC by positive selection using a magnetic cell separator (MACS; Miltenyi Biotex, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity, assessed by FACS analysis using a FITC-labeled anti-CD13 mAb (Cymbys, Hants, UK), was >95%. Monocytes were cultured in culture medium (CM) consisting of RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, and 0.1 mM nonessential amino acids (all from Life Technologies, Cergy Pontoise, France) at 5×10^6 cells/5 ml/well in 6-well tissue culture plates (Costar, Cambridge, MA) with 20 ng/ml IL-4 and 20 ng/ml GM-CSF (R&D Systems, Abingdon, UK). On day 6, cells were analyzed by FACS, as described above; only homogeneous immature DC populations characterized by high levels of CD1a and an absence of CD83 expression were used. Monocytes and DCs were then cultured at 10^6 cells/1 ml/well in 24-well flat-bottom tissue culture plates (Costar) in CM and in cytokine-containing CM. Measurement of virus or cytokine production was performed in the supernatant of cultures set up with 2×10^6 cells/ml. In some experiments, monocytes were stimulated with 20 ng/ml GM-CSF or cultured in the presence of anti-GM-CSF or control Abs (R&D Systems) at 10 or 20 μg/ml. In other experiments, monocytes and DCs were activated with 10 ng/ml of LPS (from *Escherichia coli* isotype

0111:B4; Sigma Chemical Co., St. Louis, MO); maturation of DCs was induced by incubation with 20 ng/ml TNF-α (R&D Systems).

2. Virus preparation and infection

PIV3 (American Type Culture Collection (ATCC, Rockville, MD, VR-93) was grown and propagated in Vero cells (African green monkey kidney) in medium (DMEM, 2 mM L-glutamine, 1 μg/ml gentamicin, 2.5% $NaHCO_2$, 5% SVF, Life Technologies) and purified on a sucrose gradient. Inactivated PIV3 was obtained after UV irradiation for 45 min at 1 302.8 mW/cm^2. Monocytes and DCs were infected for 2 h at 37°C with PIV3, inactivated PIV3, or CM at a m.o.i. from 1 to 10. Cells were washed extensively to eliminate the remaining virus and cultured at 37°C in CM. Virus production in cell-free supernatant was assessed by titration on Vero cell cultures, with titers being calculated according to the Kärber method (Dougherty, 1964). Data are expressed as $\log_{10}$ tissue culture infectious $dose_{50\%}$ ($TCID_{50}$)/ml.

3. Flow cytometric measurement of surface Ag expression

FACS analysis was performed using a FACSvantage cytofluorometer (Becton Dickinson, Erembodegem, Belgium) with the following mAbs: FITC-labeled anti-CD1a (Immunoquality Products, Groningen, The Netherlands), anti-CD80 and anti-CD86 (both from Pharmingen, San Diego, CA), and anti-CD54 and anti-HLA-DR (both from Becton Dickinson). The binding of the anti-CD83 mAb (Immunotech, Marseille, France) was revealed by FITC-labeled anti-mouse IgG Ab (Silenus, Hauworth, Australia). Expression of viral antigens on the cell surface was determined by incubation with polyclonal immune sera (1/200) obtained from BALB/c mice (purchased from IFFA CREDO, l'Arbresle, France) immunized by 3 ip injections either with 100 μl of purified PIV3 with complete Freund adjuvant (50% v/v, Sigma) or, for the control mouse antiserum, with 20 μg of P40, which is the outer membrane protein A (OMPA) of *Klebsiella pneumoniae* (Rauly *et al.,* 1999). FITC-labeled anti-mouse IgG Ab was used as a secondary reagent. Control isotype mAbs were from Dako (Glostrup, Denmark). Cells were analyzed using Cellquest software after removing debris and staining dead cells with propidium iodide (PI). Results are expressed in mean fluorescence intensity (MFI) values, as the percentage of positive cells after subtraction of the MFI, or the percentage obtained with the control mAb.

4. Determination of cell viability and apoptosis

Enumeration of viable cells was performed using the trypan blue dye exclusion method. Cell apoptosis was determined using the FITC-labeled annexin V kit (Immunotech) according to the manufacturer's recommendations. Briefly, cell samples dispensed in a V-bottom 96-well plate were centrifuged and resuspended in 200 μl of binding buffer in the presence of 3 μl of FITC–annexin V and 3 μl of PI. After a 10-min incubation on ice, cell samples were analyzed by flow cytometry. Annexin V^{neg}-PI^{neg} cells represent living cells, annexin V^{pos}-PI^{neg} cells represent early apoptotic cells, and annexin V^{pos}-PI^{pos} cells define late apoptotic and secondary necrotic cells.

5. Quantification of bioactive IL-10 and IL-12

DCs were incubated for 48 h with various stimuli, as described above, and IL-10 and the biologically active IL-12 p40/p35 heterodimer (IL-12 p75) were measured in cell-free culture supernatants. IL-10 was quantified by ELISA using a commercial kit (R&D Systems) according to the manufacturer's recommendations (sensitivity of 0.5 pg/ml). The concentration of the biologically active IL-12 p40/p35 heterodimer (IL-12 p75) was determined in cell-free culture supernatants by flow cytometry, using the Quantiflow IL-12 Immunoassay kit (Bioergonomics, St. Paul, MN) (sensitivity, 5 pg/ml). Briefly, 100 μl of culture supernatants was dispensed in a V-bottom 96-well plate. Two microliters of capture beads (anti-IL-12 antibody-coated beads) was added per well and the plate was incubated for 2 h at RT with orbital agitation. In parallel, a standard curve was set up by adding 100 μl of IL-12 standards into wells. Beads were then washed three times by adding 150 μl of 1× the IFA buffer provided with the kit. Plates were decanted and 2 μl of PE-conjugated IL-12 reporter antibody was added per well. After 1 h of incubation at RT with orbital agitation, beads were washed three times with IFA buffer, transferred into FACS tubes, and analyzed by flow cytometry. The fluorescent signal of the beads was collected in log scale and the settings of the PE channel photomultiplier were adjusted by running a blank bead provided in the kit. The mean channel fluorescent signal of PE was recorded. A standard curve was built by plotting mean channel fluorescence versus IL-12 concentration, for IL-12 standards. The concentration of IL-12 in experimental samples was determined by comparing the mean channel fluorescence obtained with the standard curve. The IL-12 concentrations of samples are given in pg/ml.

6. Analysis of viral RNA and mRNA expression by PCR

Briefly, cells were resuspended in 1 ml TRIzol reagent (Life Technologies). After extraction with chloroform, total RNA was precipitated by isopropyl alcohol. First-strand cDNA was synthesized from 2 μg of total RNA by reverse transcription using an oligo(dT) primer and reverse transcriptase (Promega, Madison, WI). PCR amplification was performed with an amount of cDNA corresponding to 25 ng of starting total RNA (2 min at 94°C followed by 30 cycles (30 s at 94°C, 1 min at 60°C, and 1 min at 72°C) and then followed by a final extension of 4 min at 72°C).

The sequences of the specific oligonucleotides were 5′-ATGTGGCTGCAGAGCCTGCTGCTC-3′ and 5′-TCACTCCTGGACTGGCTCCCAGCAG-3′ for GM-CSF and 5′-CATGGATGATGATATCGCCG-3′ and 5′-GCTGGAAGGTGGACAGCGAG-3′ for β-actin. The PCR products were analyzed on a 1% agarose gel by electrophoresis in the presence of ethidium bromide.

7. Primary allogenic mixed lymphocyte reaction

DCs were washed and incubated with culture medium, PIV3, or inactivated PIV3 at a m.o.i. of 5 or 10. After washing, these cells were recultured at 2×10^6 cells/2 ml/well in 6-well culture plates in cytokine-containing CM. Control cells for maturation were either unstimulated or stimulated with 20 ng/ml TNF-α. On day 2 of the culture, DCs were washed twice in CM, UV-irradiated (0.25 J/cm^2), and cultured in quadruplicate wells at 5×10^4 cells/200 μl/well in 96-well flat-bottom culture plates with 2×10^5 allogenic T cells. T cells were purified from PBMCs of healthy volunteers by rosetting with sheep red blood cells. The purity of T cells assessed by FACS analysis using a FITC-labeled anti-CD3 mAb (Immunotech) was >95%. After 3 or 5 days, cells were pulsed during the last 16 h with [^{3}H]thymidine (0.25 μCi/well) (Amersham, Amersham, UK) and harvested, and thymidine incorporation was counted using a Packard 1900CA-Tricarb β-counter (Packard Instrument, Meriden, CT). Results are expressed in cpm (means ± SD) of quadruplicate values.

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